# Sarcolemmal mechanisms for $pH_i$ recovery from alkalosis in the guinea-pig ventricular myocyte

Chae-Hun Leem and Richard D. Vaughan-Jones

*University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK*

(Received 22 July 1997; accepted after revision 10 February 1998)

1. The mechanism of $pH_i$ recovery from an intracellular alkali load (induced by acetate prepulse or by reduction/removal of ambient $P_{CO_2}$) was investigated using intracellular SNARF fluorescence in the guinea-pig ventricular myocyte.

2. In Hepes buffer ($pH_o$ 7·40), $pH_i$ recovery was inhibited by removal of extracellular $Cl^-$, but not by removal of $Na_o^+$ or elevation of $K_o^+$. Recovery was unaffected by the stilbene drug DIDS (4,4-diisothiocyanatostilbene-disulphonic acid), but was slowed dose dependently by the stilbene drug DBDS (dibenzamidostilbene-disulphonic acid).

3. In 5% $CO_2/HCO_3^-$ buffer ($pH_o$ 7·40), $pH_i$ recovery was faster than in Hepes buffer. It consisted of an initial rapid recovery phase followed by a slow phase. Much of the rapid phase has been attributed to $CO_2$-dependent buffering. The slow phase was inhibited completely by $Cl_o^-$ removal but not by $Na_o^+$ removal or $K_o^+$ elevation.

4. At a test $pH_i$ of 7·30 in $CO_2/HCO_3^-$ buffer, the slow phase was inhibited 70% by DIDS. The mean DIDS-inhibitable acid influx was equivalent in magnitude to the $HCO_3^-$-stimulated acid influx. Similarly, the DIDS-*insensitive* influx was equivalent to that estimated in Hepes buffer.

5. We conclude that two independent sarcolemmal acid-loading carriers are stimulated by a rise of $pH_i$ and account for the slow phase of recovery from an alkali load. The results are consistent with activation of a DIDS-sensitive $Cl^-$–$HCO_3^-$ anion exchanger (AE) to produce $HCO_3^-$ efflux, and a DIDS-insensitive $Cl^-$–$OH^-$ exchanger (CHE) to produce $OH^-$ efflux. $H^+$–$Cl^-$ co-influx as the alternative configuration for CHE is not, however, excluded.

6. The dual acid-loading system (AE plus CHE), previously shown to be activated by a fall of extracellular pH, is thus activated by a rise of intracellular pH. Activity of the dual-loading system is therefore controlled by pH on both sides of the cardiac sarcolemma.

Intracellular pH ($pH_i$) exerts considerable influence on cardiac contractility and rhythm (Orchard & Kentish, 1990; Orchard & Cingolani, 1994). It is controlled in mammalian cardiac cells by means of sarcolemmal acid-extrusion and acid-loading carriers. The acid-equivalent extrusion carriers are $Na^+$–$H^+$ exchange (Deitmer & Ellis, 1980) and $Na^+$–$HCO_3^-$ symport (Dart & Vaughan-Jones, 1992; Lagadic-Gossmann, Buckler & Vaughan-Jones, 1992, but cf. Liu, Piwnica-Worms & Lieberman, 1990) while the loading carriers are $Cl^-$–$HCO_3^-$ exchange (Vaughan-Jones, 1979; Xu & Spitzer, 1994) and a novel $Cl^-$-dependent carrier, proposed to be $Cl^-$–$OH^-$ exchange (Sun, Leem & Vaughan-Jones, 1996). We have shown recently that, in mammalian ventricular myocytes, the two sarcolemmal acid loaders are stimulated by a fall of extracellular pH, and that this stimulation accounts for the subsequent fall of $pH_i$ (Sun *et al.* 1996). In addition to this extracellular pH sensitivity, earlier reports showed that $Cl^-$–$HCO_3^-$ exchange, one of the two acid loaders, is stimulated by a rise of *intracellular* pH, leading to $pH_i$ recovery from an alkali load (Vaughan-Jones, 1982; Xu & Spitzer, 1994). The possibility, however, of high $pH_i$ activation of $Cl^-$–$OH^-$ exchange has not so far been investigated.

In the preceding paper (Leem & Vaughan-Jones, 1998), we showed that much of the initial rapid phase of $pH_i$ recovery from an intracellular alkali load is due to slow $CO_2$-dependent buffering. In the present work, we examine the contribution to $pH_i$ recovery made by the acid loaders. In particular, we investigate the possible role played by the novel $Cl^-$–$OH^-$ exchanger (CHE) . The high $pH_i$ stimulation of $Cl^-$–$HCO_3^-$ anion exchange (AE) is also re-assessed, since its contribution to $pH_i$ recovery has not previously been distinguished from that of CHE.

Preliminary reports of this work have appeared (Leem, Loh & Vaughan-Jones, 1996; Leem & Vaughan-Jones, 1996).

## METHODS

Details are given in full in the Methods of the preceding paper (Leem & Vaughan-Jones, 1998). Briefly, ventricular myocytes were isolated enzymically from hearts of albino guinea-pigs (killed by cervical dislocation) weighing 350–450 g. Intracellular pH was recorded ratiometrically from single myocytes, AM-loaded with carboxy SNARF-1. Unless otherwise stated, cells were superfused with either Hepes buffer (20 mM) or 5% $CO_2$–22 mM $HCO_3^-$-buffered Tyrode solution, pH 7·40 at 37 °C. Intracellular alkali loads were induced by acetate prepulsing (40–80 mM) or by reduction/removal of $P_{CO_2}$ at $pH_o$ 7·40, as specified. Composition of solutions is also given in the Methods of the preceding paper (Leem & Vaughan-Jones, 1998). All drugs and chemicals were obtained from Sigma apart from DIDS (diisothiocyanatostilbene disulphonic acid) which was from Boehringer Mannheim and Hoe 694 which was a gift from Dr U. Albus, Hoechst Akitengesellschaft (Germany). These drugs were added as the solid to solutions shortly before use. DIDS solutions were protected from light and used for no more than 4 h.

### Calculation of sarcolemmal acid-equivalent flux

This was calculated from the $pH_i$ record as: $J_H = \beta_{tot} \times dpH_i/dt$, where $J_H$ is net sarcolemmal flux, and $\beta_{tot}$ (total intracellular buffering power) = [$\beta_i$ (intrinsic buffering power) + $\beta_{CO_2}$ ($CO_2$-dependent buffering power)]. When superfusates are Hepes buffered, $\beta_{CO_2} = 0$, and thus $\beta_{tot} = \beta_i$. The $\beta_i$ at any given $pH_i$ was estimated from the equation, determined previously by Lagadic-Gossmann *et al.* (1992): $\beta_i = -28\,pH_i + 222{\cdot}6$. The $\beta_{CO_2}$ at any given $pH_i$ was determined from the equation: $\beta_{CO_2} = 2{\cdot}3[HCO_3^-]_i$, where $[HCO_3^-]_i$ is the intracellular concentration of bicarbonate anion, assuming $CO_2$ is at equilibrium across the sarcolemma, and assuming that intracellular $CO_2$ hydration is also at equilibrium. $[HCO_3^-]_i$ was calculated from a rearrangement of the Henderson–Hasselbalch equation:

$$[HCO_3^-]_i = [HCO_3^-]_o \times 10^{(pH_i - pH_o)},$$

where $[HCO_3^-]_o$ is the bicarbonate concentration of the extracellular solution. This assumes that the $CO_2$ solubility coefficient and the apparent p$K$ of $CO_2$ hydration do not vary between the extracellular and intracellular fluid. The previous paper (Leem & Vaughan-Jones, 1998) showed that $CO_2$ hydration is out of equilibrium (OOE) for periods of up to 2·5 min following the imposition of an intracellular alkali load (by weak acid prepulse). Estimates of sarcolemmal acid flux were therefore always made > 2·5 min following such a prepulse.

Rates of change of $pH_i$ ($dpH_i/dt$) at any given $pH_i$ were obtained by computer from the first time differential of the best-fit polynomial equation (Sigmaplot, Jandel Corp.) to experimental data points sampled at 0·5 s intervals. The computer fit was assessed by comparing it with the original data using Student's paired $t$ test (acceptable if $P > 0{\cdot}99$).

All statistical data were expressed as means ± S.E.M.

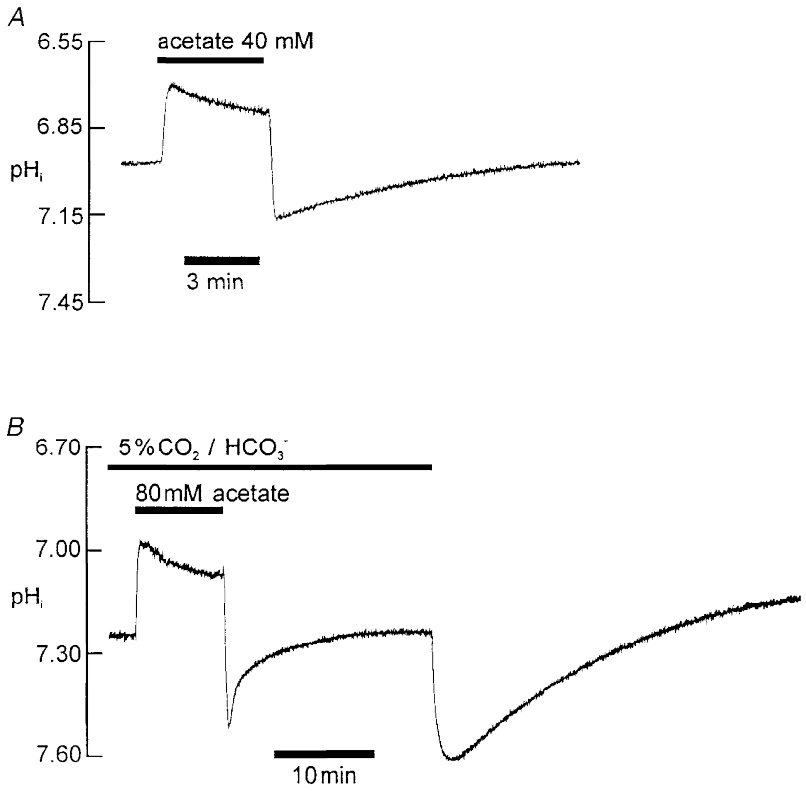


**Figure 1. Intracellular pH recovery from an alkali load**

Extracellular pH 7·40 throughout. *A*, intracellular alkalosis induced by 40 mM acetate prepulse in Hepes-buffered solution. Trace shows ratiometric SNARF recording of $pH_i$ in an isolated ventricular myocyte. *B*, comparison of time course of $pH_i$ recovery from alkalosis in presence (first section of $pH_i$ recording) and absence (second section of $pH_i$ recording) of 5% $CO_2/HCO_3^-$ buffer. First alkali load induced by 80 mM acetate prepulse; second alkali load induced by replacement of 5% $CO_2$–22 mM $HCO_3^-$ buffer by 20 mM Hepes.

## RESULTS

### Intracellular pH recovery from an alkali load

Figure 1*A* illustrates $pH_i$ recovery from an intracellular alkali load, observed in the nominal absence of $CO_2/HCO_3^-$ (Hepes buffer). A rise in $pH_i$ of about 0·25 units was induced by using the acetate prepulse technique. The $pH_i$ subsequently recovered ($n = 42$). A similar recovery in Hepes-buffered medium is evident in the later part of the trace shown in Fig. 1*B*. In this case the alkali load was induced by switching from $CO_2/HCO_3^-$ to Hepes buffer. An identical result was observed in six other cells. A similar recovery in Hepes buffer was also seen following an alkali load induced by a 40 mM propionate prepulse ($n = 6$; not shown). The property of $pH_i$ recovery is therefore independent of the method of alkali loading.

Figure 1*B* compares $pH_i$ recovery from alkalosis in $CO_2/HCO_3^-$ buffer (induced by acetate prepulse) with that seen in Hepes buffer (induced by $CO_2$ removal). The initial $pH_i$ recovery was considerably faster in $CO_2/HCO_3^-$ buffer ($n = 43$). In the preceding paper (Leem & Vaughan-Jones, 1998) we showed that much of this initial recovery (< 2·5 min after acetate removal) is caused by $CO_2$-dependent buffering. Inspection of the trace shown in Fig. 1*B* reveals that the time course of the later phase of $pH_i$ recovery in $CO_2/HCO_3^-$ medium (> 2·5 min after acetate removal) also differs from that seen in Hepes buffer. A comparison of rates, however, should take into account the different intracellular buffering power in the two situations, as detailed later in the Results. The $pH_i$ in Fig. 1*B* stabilized more quickly in $CO_2/HCO_3^-$ ($n = 7$), and at a slightly more alkaline $pH_i$. Variable differences of steady-state $pH_i$ in $HCO_3^-$ *versus* Hepes-buffered solutions have been reported extensively for ventricular cells (e.g. Bountra, Powell & Vaughan-Jones, 1990; Blank *et al.* 1992; Sun *et al.* 1996), and so are not analysed in detail here. Figure 1*B* illustrates that $CO_2/HCO_3^-$ influences both the early (rapid) and the late (slower) phases of $pH_i$ recovery from alkalosis.

In the remainder of the Results, we examine the ionic dependence and drug sensitivity of the slow phase of $pH_i$ recovery from alkalosis, while also comparing data obtained in the presence and nominal absence of $CO_2/HCO_3^-$.

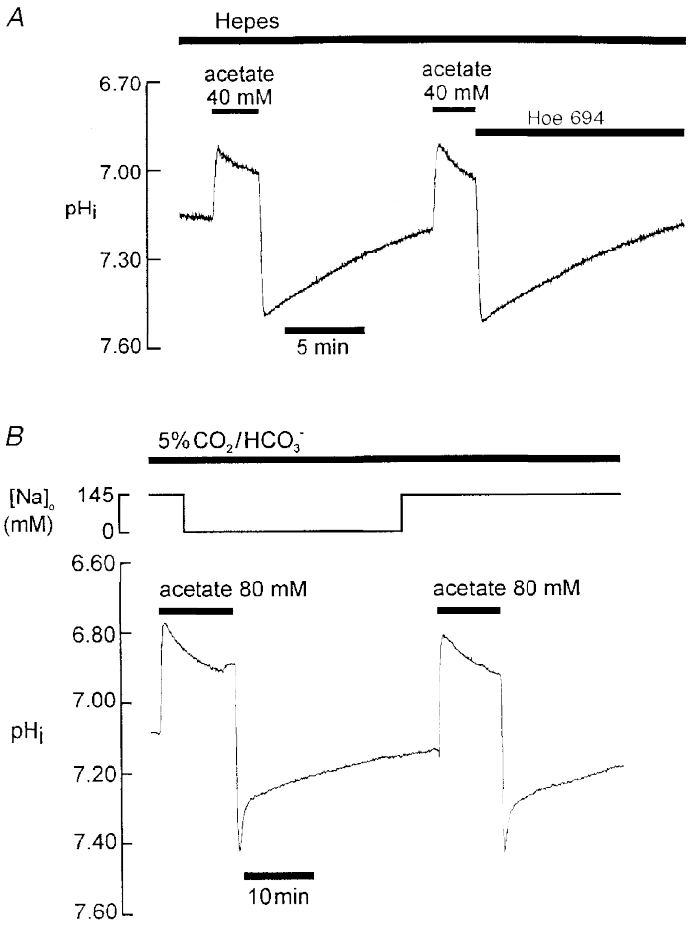


**Figure 2. Recovery from alkali load is independent of $Na^+$–$H^+$ exchange and of $Na_o^+$**

$pH_o$ 7·40 throughout. *A*, Hepes buffer; application of 30 μM Hoe 694 (an $Na^+$–$H^+$ exchange (NHE)-1 inhibitor) indicated by bar above trace *B*, 5% $CO_2$–22 mM $HCO_3^-$ buffer; $Na_o^+$ replaced by NMDG as indicated at top of figure.

**Effect of Hoe 694 and of $Na_o^+$ removal**

Hoe 694 is a high-affinity inhibitor of cardiac $Na^+$–$H^+$ exchange (Scholz, Albus, Lang, Martorana, Englert & Scholkens, 1993; Loh, Sun & Vaughan-Jones, 1996). Figure 2*A* shows that in Hepes buffer, $pH_i$ recovery from alkalosis was unimpaired by 30 $\mu$M Hoe 694, a dose sufficient to inhibit cardiac $Na^+$–$H^+$ exchange maximally (Loh *et al.* 1996). In a total of five experiments, $pH_i$ recovered at a rate of $0{\cdot}0202 \pm 0{\cdot}003$ pH units $min^{-1}$ in the presence of 30 $\mu$M Hoe, compared with $0{\cdot}019 \pm 0{\cdot}004$ pH units $min^{-1}$ under control conditions (measured at $pH_i$ 7·31 in both cases; no significant difference, $P > 0{\cdot}05$, paired $t$ test). This indicates that modulation of $Na^+$–$H^+$ exchange activity is not responsible for the recovery. This conclusion is reinforced by the observation (not illustrated) that recovery was not impaired by complete removal of extracellular $Na^+$ (replaced isosmotically by *N*-methyl-D-glucamine (NMDG)) a manoeuvre which, like Hoe 694 addition, inhibits $Na^+$–$H^+$ exchange (note that significant reversal of $Na^+$–$H^+$ exchange in $Na^+$-free solution does not occur in the ventricular myocyte; see, for example, Loh *et al.* 1996): recovery rate at $pH_i$ 7·28 was $0{\cdot}027 \pm 0{\cdot}004$ pH units $min^{-1}$ in $Na^+$-free solution compared with $0{\cdot}022 \pm 0{\cdot}003$ pH units $min^{-1}$ under control conditions ($n = 5$; $P > 0{\cdot}05$, paired $t$ test)

$Na_o^+$ removal (replaced by NMDG) also had no inhibitory effect on $pH_i$ recovery observed in the presence of $CO_2/HCO_3^-$ buffer (Fig. 2*B*; $n = 5$; recovery rate (slow, late phase) at $pH_i$ 7·23 in $Na^+$-free conditions was $0{\cdot}145 \pm 0{\cdot}001$ pH units $min^{-1}$ compared with $0{\cdot}141 \pm 0{\cdot}003$ pH units $min^{-1}$ in control conditions; $P > 0{\cdot}05$, paired $t$ test). Thus, neither the early (Leem & Vaughan-Jones, 1998) nor the late phase of recovery in $CO_2/HCO_3^-$ buffer is $Na_o^+$ dependent. It should be noted that $Na_i^+$ in cardiac cells declines within a couple of minutes to levels $\ll 1$ mM following $Na_o^+$ removal (Ellis, 1977) thus also excluding a dependence on intracellular $Na^+$.

**Replacing $Na_o^+$ with $K^+$.** Recovery of $pH_i$ (from a 40 mM acetate prepulse) was also unimpaired by isosmotic replacement of $Na^+$ with $K^+$ (instead of NMDG). This was the case in both Hepes buffer and $CO_2/HCO_3^-$-buffered solution (not illustrated; recovery rate not significantly different when measured at $pH_i$ 7·36 in Hepes buffer, in 4·5

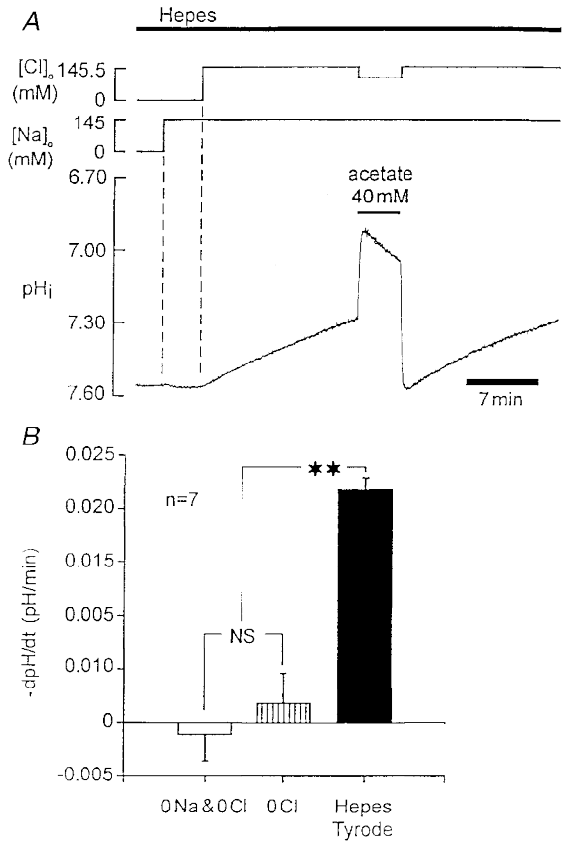


**Figure 3. Recovery in Hepes buffer is $Cl_o^-$ dependent**

*A*, trace begins after cell has been exposed to $Cl^-$-free (gluconate substituted), $Na^+$-free (NMDG substituted) solution for about 10 min. An acetate prepulse (40 mM) was used to raise $pH_i$ to 7·55. Note lack of $pH_i$ recovery. Re-addition of $Na_o^+$ and then $Cl_o^-$ is indicated at the top of the figure. *B*, histogram showing $pH_i$ recovery rate (measured at $pH_i$ $7{\cdot}38 \pm 0{\cdot}03$, mean ± S.E.M.) in normal Hepes–Tyrode solution (black column), $Cl^-$-free Tyrode solution (hatched column) and $Na^+$-free, $Cl^-$-free Tyrode solution. $pH_o$ 7·40 throughout. NS, not significant, $P > 0{\cdot}05$, Student's paired $t$ test; ** significant difference, $P < 0{\cdot}00005$.

and 144·5 mM $K_o^+$, $P > 0{\cdot}05$, paired $t$ test, $n = 5$; recovery rate not significantly different in $CO_2/HCO_3^-$ buffer in 4·5 and 144·5 mM $K_o^+$, measured at $pH_i$ 7·22, $n = 3$, $P > 0{\cdot}05$, paired $t$ test). Since membrane potential is depolarized to approximately 0 mV in the high $K^+$ solution, this suggests that the recovery mechanism is voltage insensitive, at least in the physiological range of membrane potential.

### Effect of $Cl_o^-$ removal

**Hepes buffer**. Recovery was abolished entirely by removal of extracellular chloride (replaced by gluconate and/or glucuronate). Figure 3*A* shows an experiment where $pH_i$ had initially been elevated by using an acetate (40 mM) prepulse. $Na_o^+$ and $Cl_o^-$ had also been removed (see legend for details). Intracellular pH was stable at 7·55, showing no tendency to move towards less alkaline levels. Figure 3 shows that re-adding $Na_o^+$ had no effect on $pH_i$ which remained at the elevated level of 7·55. Re-adding $Cl_o^-$, however, prompted an immediate recovery at a rate identical to that seen in the control recovery observed in the last part of the experiment. Figure 3*B* shows pooled data from seven such experiments, showing that recovery is inhibited equally well by removal of $Na_o^+$ plus $Cl_o^-$, or by removal of $Cl_o^-$ alone. This (i) confirms that recovery is not $Na^+$ dependent and (ii) demonstrates an absolute dependence of recovery on $Cl_o^-$.

**$CO_2/HCO_3^-$ buffer**. Under these conditions, $Cl^-$–$HCO_3^-$ exchange is functional, and would be expected to contribute to $pH_i$ recovery (Vaughan-Jones, 1982; Xu & Spitzer, 1994). The traces shown in Fig. 4 show that recovery was again abolished in the absence of extracellular $Cl^-$. Two different methods of alkali loading are illustrated, the acetate prepulse (simulating metabolic alkalosis; Fig. 4*A*; same result in 21 experiments) and reduction of $P_{CO_2}$ (simulating respiratory alkalosis; $P_{CO_2}$ reduction from 10 to 5%; Fig. 4*B*; same result in 17 experiments). Note that, in Fig. 4*A*, an initial rapid phase of $pH_i$ recovery following acetate removal was evident in $Cl^-$-free solution (Leem & Vaughan-Jones, 1998) even though the slow phase was abolished ($n = 21$). This is consistent with $CO_2$-dependent buffering contributing significantly to the rapid but not the slow phase.

We conclude that the sarcolemmal acid-loading mechanisms active in Hepes or $CO_2/HCO_3^-$ buffer are $Cl_o^-$ dependent.

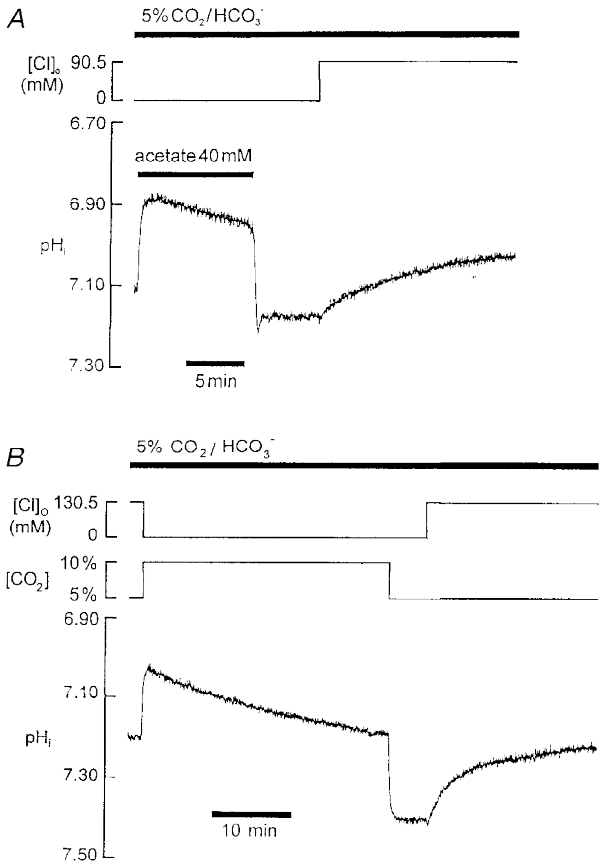


**Figure 4. Slow recovery phase in $CO_2/HCO_3^-$ buffer is also $Cl_o^-$ dependent**

Two types of alkali load are investigated, simulated metabolic (*A*) and respiratory (*B*) alkalosis. *A*, acetate pre-pulsing to raise $pH_i$. Note slow recovery phase in $Cl^-$-free solution on $Cl_o^-$ re-admission; in this case only 90·5 mM $Cl_o^-$ re-admitted (remaining anion balance being gluconate) *B*, $P_{CO_2}$ was elevated to 10% (extracellular bicarbonate raised to 44 mM; constant $pH_o$ of 7·40) for several minutes and then reduced back to 5% (22 mM $HCO_3^-$) to induce intracellular alkalosis. Recovery only occurred upon readmission of $Cl_o^-$.

### Effect of stilbenes

Recovery of $pH_i$ from an alkali load in heart has been attributed previously to activation of $Cl^-$–$HCO_3^-$ exchange (AE). Figure 5*A* shows that 500 $\mu$M DIDS, a high-affinity AE inhibitor (Cabantchik, Knauf & Rothstein, 1978), had no effect on $pH_i$ recovery in Hepes-buffered solution ($n = 6$, 0·1–0·5 mM DIDS), but it slowed considerably the recovery observed in $CO_2/HCO_3^-$-buffered solution, notably during the secondary recovery phase (Fig. 5*B*; $n = 7$; 0·1–0·5 mM DIDS). The effect of DIDS is analysed quantitatively later in the Results.

The trace shown in Fig. 6*A* shows that another stilbene, dibenzamidostilbene-disulphonic acid (DBDS), which slows the DIDS-insensitive $Cl^-$–$OH^-$ exchanger (CHE) (Sun *et al.* 1996), also slowed $pH_i$ recovery in Hepes buffer. Slowing was dose dependent in the range up to 300 $\mu$M DBDS, which is close to the maximum solubility of the drug. At this concentration, DBDS inhibited recovery by 60%, which is similar to its inhibition (70%) of low $pH_o$-activated CHE (Sun *et al.* 1996). The effect of DBDS on recovery in $CO_2/HCO_3^-$ buffer was not tested.

### Dual acid loading mediates $pH_i$ recovery

The trace shown in Fig. 7*A* compares $pH_i$ recovery from alkalosis in $CO_2/HCO_3^-$ buffer in the presence and absence of DIDS, with recovery in Hepes buffer. As before, the secondary recovery in $CO_2/HCO_3^-$ buffer was slowed by DIDS, and it was slowed following a switch from $CO_2/HCO_3^-$ to Hepes buffer. The left-hand couplet of the histogram in Fig. 7*B* summarizes data from several experiments where recovery rate was compared, at a common $pH_i$ (7·30), in Hepes and bicarbonate buffer (without any intermediate exposure to DIDS). Note that all rate measurements in $CO_2/HCO_3^-$ buffer were made > 2 min following acetate removal, to permit out-of-equilibrium buffer conditions to subside. Net acid influx was stimulated nearly 3-fold in $CO_2/HCO_3^-$ compared with Hepes buffer. Comparison of the left- and right-hand couplets of Fig. 7*B* shows that reduction of net acid loading caused by removal of $CO_2/HCO_3^-$ (70% decrease) is virtually identical to the reduction caused by addition of DIDS in the presence of $CO_2/HCO_3^-$ buffer. Thus all $HCO_3^-$-stimulated acid loading must be DIDS inhibitable. In contrast, acid loading in $HCO_3^-$-free buffer (Hepes) is equivalent to the DIDS-resistant acid loading

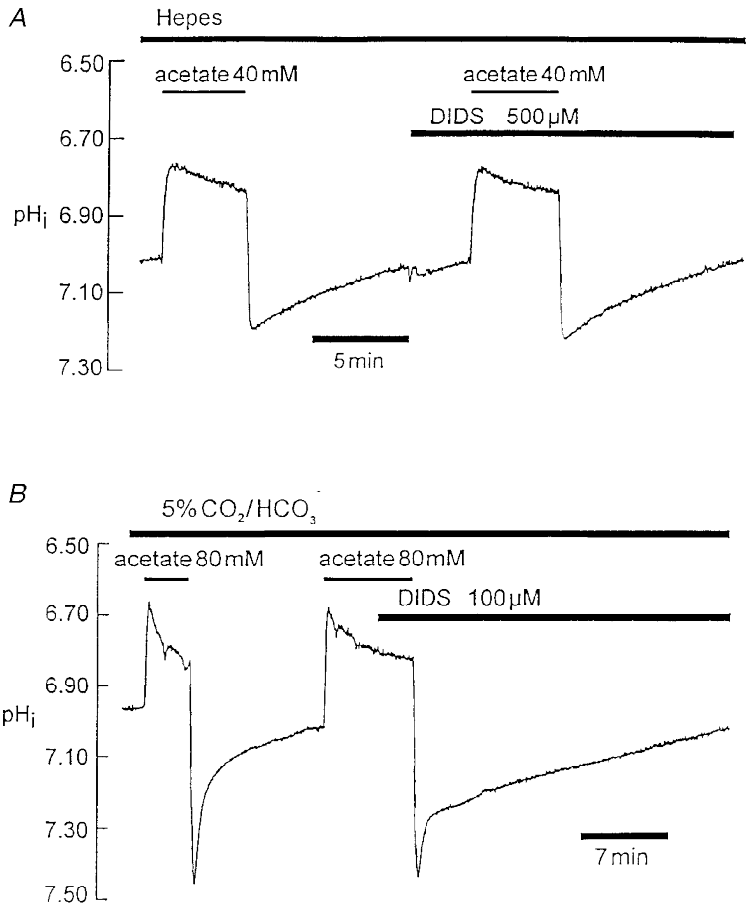


**Figure 5. DIDS attenuates the slow phase of recovery in $CO_2/HCO_3^-$ but not in Hepes buffer**

*A*, Hepes buffer; acetate prepulsing with and without 0·5 mM DIDS. *B*, $CO_2/HCO_3^-$ buffer; acetate prepulsing with and without 100 $\mu$M DIDS.

observed in $CO_2/HCO_3^-$ buffer. As discussed below, these results are consistent with the presence of two independent acid-loading mechanisms in the mammalian cardiac cell.

## DISCUSSION

### Intracellular pH recovery from alkalosis: role for AE and CHE

The present work indicates that the slow phase of $pH_i$ recovery from an alkali load in the cardiac cell is mediated through two independent, sarcolemmal acid-loading carriers, one $HCO_3^-$ dependent and DIDS sensitive, the other $HCO_3^-$ independent and DIDS insensitive. Both carriers are independent of $Na^+$ but dependent on $Cl_o^-$, and appear to be insensitive to membrane depolarization ($K_o^+$ induced). These results are consistent with the dual acid-loading mechanism proposed recently for the ventricular myocyte (Sun *et al.* 1996). This comprises a DIDS-sensitive $Cl^-$–$HCO_3^-$ exchanger (AE) and a DIDS-insensitive but DBDS-inhibited $Cl^-$–$OH^-$ exchanger (CHE).

While we have not entirely eliminated the possibility that $pH_i$ recovery in Hepes buffer (putative CHE) is dependent on trace levels of $HCO_3^-$ generated from metabolic or atmospheric $CO_2$ (rather than upon $OH^-$), its similarity with acid loading observed previously (Sun *et al.* 1996) during CHE stimulation by low $pH_o$ (Hepes buffer), is consistent with the same mechanism. In the latter case, the putative CHE component was not dependent on $HCO_3^-$ since it continued to function as an acid loader in the complete absence of metabolic or atmospheric $CO_2$ (Leem & Vaughan-Jones, 1997). This result, however, while excluding $HCO_3^-$, does not distinguish between $H^+$ and $OH^-$ as the transported species (see Sun *et al.* 1996).

When combined with the results of the preceding paper (Leem & Vaughan-Jones, 1998), we see that $pH_i$ recovery from an alkali load is a combination of two processes. The first is buffering, and here intrinsic buffering is essentially instantaneous (at least on the present time scale of experiments) while $CO_2$-dependent buffering is notably slow. The second process is acid equivalent influx through sarco-

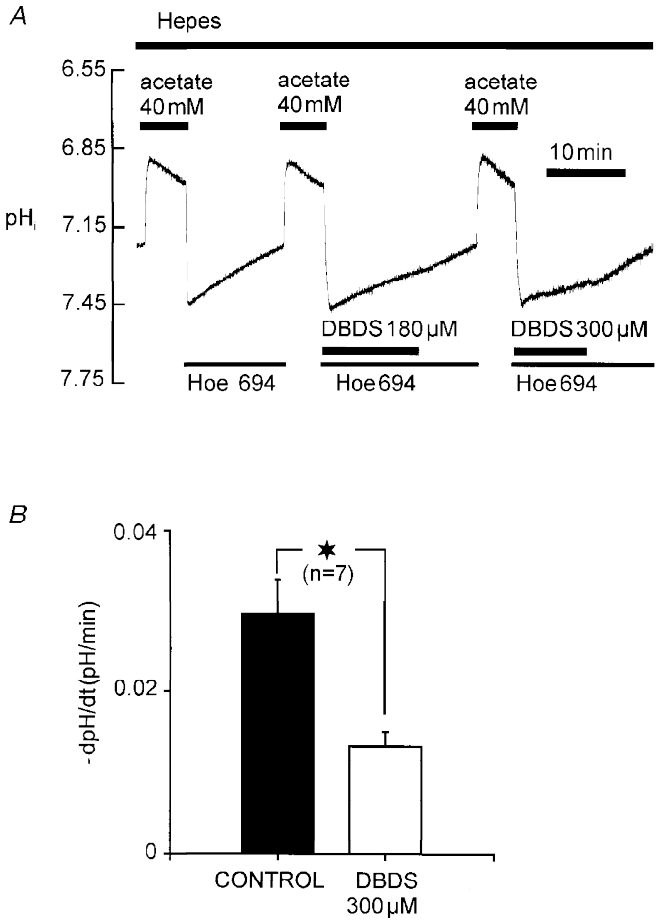


**Figure 6. DBDS blocks recovery in Hepes buffer dose dependently**

*A*, experimental protocol; Hoe 694 applied to remove possible influence on $pH_i$ recovery from $Na^+$–$H^+$ exchanger. *B*, histogram comparing mean $pH_i$ recovery rate recorded at $pH_i$ 7·43 ± 0·015 (mean ± S.E.M.), with and without 300 $\mu$M DBDS (close to maximum solubility of drug).

lemmal carriers. Although these carriers must contribute to both the early and late phases of $pH_i$ recovery, their initial participation is hard to quantify from the $pH_i$ record, because at this time the $CO_2/HCO_3^-$ buffer is out of equilibrium (Leem & Vaughan-Jones, 1998). Once the out-of-equilibrium period is over, $pH_i$ recovery occurs only through sarcolemmal transport and it is here, during the slow recovery phase, that carrier activity can be readily quantified.

### The $pH_i$ dependence of acid loading

The equilibrium $pH_i$ for both AE and CHE carriers is approximately 6·70 (Sun *et al.* 1996). Therefore, at normal steady-state $pH_i$ (about 7·10), the carriers are thermodynamically energized in favour of acid-equivalent influx. Intracellular alkalosis increases the driving force for influx and, as shown in Fig. 7*B*, influx is clearly stimulated at $pH_i$ 7·30. About 70% of this influx is mediated through AE and 30% through CHE. This is to be compared with the effect of reducing extracellular pH from 7·40 to 6·40 which also stimulates acid loading, but in this case there are roughly equal contributions from AE and CHE (Sun *et al.* 1996). The dual acid-loading system in the myocyte is therefore a major mechanism for the trafficking of acid into the cardiac cell, activated by a fall of $pH_o$ and a rise of $pH_i$.

Elucidating the full $pH_i$ dependence of acid loading will require measuring it under conditions where the acid extruders have been inhibited (any residual acid extruder activity during an alkali load may offset the slow phase of $pH_i$ recovery). In the present work the effect of the extruders ($Na^+$–$H^+$ exchange and $Na^+$–$HCO_3^-$ symport) on $pH_i$ recovery from alkalosis was not systematically eliminated, although it will have been negligible at $pH_i$ 7·30 (where our measurements were made) since recovery was similar in the presence and absence of $Na_o^+$ (Fig. 2; see also Xu & Spitzer, 1994). At less alkaline values close to normal $pH_i$, however, the influence of the extruders will become significant. For example, we have previously estimated that acid loading in Hepes buffer is about 0·06 mM $min^{-1}$ at the resting $pH_i$ level, but because this is counterbalanced by extrusion

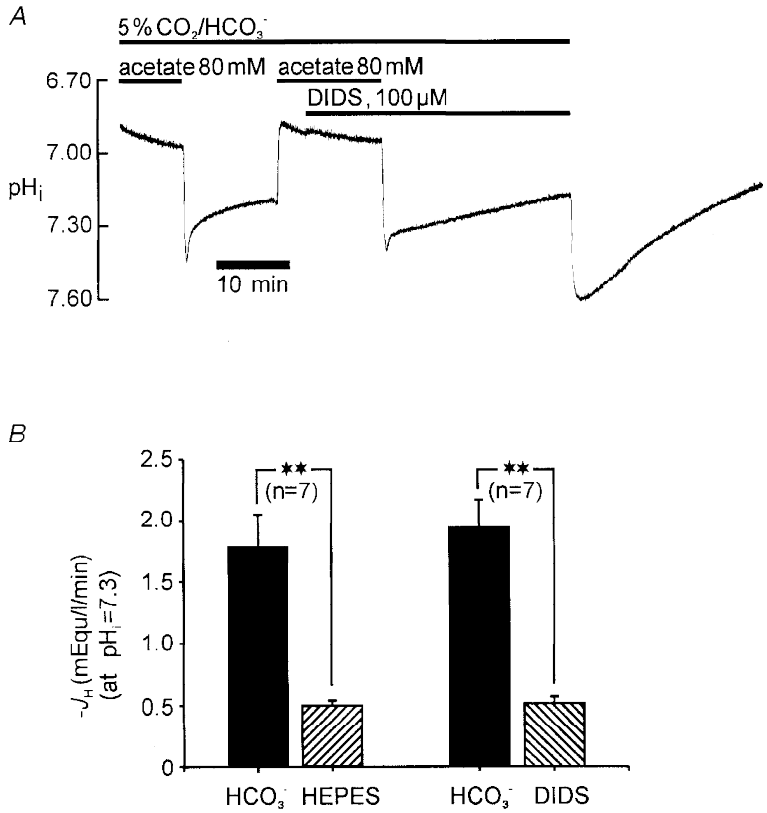


**Figure 7. DIDS-sensitive acid influx is $HCO_3^-$ dependent; DIDS-insensitive influx is $HCO_3^-$ independent**

*A*, $pH_i$ recovery from alkalosis in $CO_2/HCO_3^-$ buffer, with and without DIDS (first part of trace) should be compared with that observed in Hepes buffer (last part of trace). *B*, histograms pooling data from several experiments. Left-hand couplet compares, in the same cell ($n = 7$ cells), net acid-equivalent influx ($J_H$) measured during recovery from intracellular alkalosis at $pH_i$ 7·30 in $CO_2/HCO_3^-$ (left column) with Hepes-buffered (right column) conditions. Right-hand couplet compares, in the same cell ($n = 7$ cells), net acid influx ($J_H$) measured during recovery from intracellular alkalosis at $pH_i$ 7·30 in $CO_2/HCO_3^-$-buffered conditions in the absence (left column) and presence (right column) of 100 µM DIDS. Acid-equivalent influx ($J_H$) was calculated as described in Methods. ** Significant difference (Student's paired *t* test, $P < 0{\cdot}001$).

through $Na^+$–$H^+$ exchange, net acid loading at this $pH_i$ is zero. This emphasizes that, at steady state, both acid-extrusion and acid-loading processes exert an equal influence on $pH_i$.

Acid influx in the guinea-pig myocyte (in $CO_2/HCO_3^-$ buffer) has recently been estimated over the $pH_i$ range 7·2 to 7·6 (Xu & Spitzer, 1994). It was proposed that influx activated steeply with rising $pH_i$. In that work, however, the acid extruders were not inactivated. The $pH_i$ recovery from alkalosis was also assumed to reflect only sarcolemmal acid influx but, as pointed out in the preceding paper (Leem & Vaughan-Jones, 1998), flux measurements calculated from the $pH_i$ record shortly after establishing an alkali load will be distorted by slow $CO_2$-dependent buffering. In addition, the present work shows that recovery is by two rather than one type of sarcolemmal carrier. In future, it will therefore be important to characterize the $pH_i$ dependence of acid influx under clear buffer equilibrium conditions and under conditions where individual carrier activities can be distinguished.

The involvement of AE in $pH_i$ recovery from alkalosis is consistent with earlier findings in cardiac tissue (Vaughan-Jones, 1982; Desilets, Puceat & Vassort, 1994; Xu & Spitzer, 1994). The present work, however, is the first to identify significant contributions from an additional acid loader, CHE. Interestingly, although not commented on at the time, there is some evidence in earlier work for dual acid loading during $pH_i$ recovery (Xu & Spitzer, 1994). In addition to a bicarbonate-stimulated acid loader (AE), these authors reported a small bicarbonate-independent component of $pH_i$ recovery in the guinea-pig myocyte, and this may correspond to CHE. The evidence, however, is inconclusive since the bicarbonate-independent component appeared to be insensitive to changes of $pH_i$ and its fractional contribution to recovery was small (about 10 % at $pH_i$ 7·30). In our own work, CHE is clearly activated by a raised $pH_i$ and its contribution to recovery in $CO_2/HCO_3^-$ buffer is at least 30 % (note, however, that if the $pH_i$ sensitivity of AE and CHE differs, the precise fractional contribution from CHE will vary with $pH_i$).

### Alkali regulation in heart and the AE gene family

The molecular basis of alkali regulation in heart cannot be established until the protein sequences producing functional, wild-type cardiac $Cl^-$–$HCO_3^-$ and $Cl^-$–$OH^-$ exchange have been formally identified. One obvious possibility is that the two transporters are part of the same gene family of acid loaders, in which case $Cl^-$–$OH^-$ exchange would be a member of the AE family. There is considerable uncertainty about the AE isoform(s) responsible for alkali regulation in heart. Three principal isoforms are known (AE1–3) (Alper, 1991), and all three have been implicated at various times as being the functional $Cl^-$–$HCO_3^-$ exchanger in heart (Kudrycki, Newman & Shull, 1990; Yannoukakos, Stuart-Tilley, Fernandez, Fey, Duyk & Alper, 1994; Puceat, Korichneva, Cassoly & Vassort, 1995; Sekler, Kobayashi & Kopito, 1996). Activity of these isoforms appears to be DIDS inhibited, albeit with differing inhibition constant ($K_i$) values. CHE, however, is DIDS insensitive (at least at doses up to 0·5 mM) and $HCO_3^-$ independent (Sun *et al.* 1996; Leem & Vaughan-Jones, 1997, plus the present study). CHE may therefore represent a new DIDS-insensitive AE isoform. Alternatively the carrier may be a member of an entirely different family of acid transporters. We do not have direct proof, for example, that CHE is indeed an hydroxyl ion transporter, although its requirement for $Cl_o^-$ and its sensitivity to the stilbene DBDS is suggestive of an anion exchanger. As pointed out previously (Sun *et al.* 1996), the alternative formulation for the carrier would be an inwardly directed $H^+$–$Cl^-$ symport.

### Conclusions

Intracellular pH recovery from alkalosis in the guinea-pig cardiomyocyte is mediated through two independent acid-loading carriers, $Cl^-$–$HCO_3^-$ exchange (AE) and a putative $Cl^-$–$OH^-$ exchange (CHE). Hence the dual acid-loading system in this cell, which is stimulated by a fall of extracellular pH (Sun *et al.* 1996), is also stimulated by a rise of intracellular pH. Traffic of acid equivalents through the system is consequently regulated by pH at the extracellular and intracellular sites of both carriers. The physiological advantage to the cardiac cell of possessing two rather than one type of acid-loading carrier is unclear. What is clear, however, is that both carriers make significant contributions to sarcolemmal acid influx.

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**Acknowledgements**

We thank Ms Anna Clark for excellent technical assistance during experiments and for helping to prepare the figures. This work was funded by a grant (to R.D.V.-J.) from the British Heart Foundation.

**Corresponding author**

R. D. Vaughan-Jones: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK.

Email: Richard.Vaughan-Jones@physiol.ox.ac.uk

**Present address**

C.-H. Leem: Department of Physiology, University of Ulsan, College of Medicine, 388-1, Songpa-Ku, Poongnap-Dong, Seoul 138-040, Korea.